

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/60, 9/88, C12P 19/02, C07H 3/10		A1	(11) International Publication Number: WO 96/12026
			(43) International Publication Date: 25 April 1996 (25.04.96)
(21) International Application Number: PCT/EP95/02172		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(22) International Filing Date: 6 June 1995 (06.06.95)			
(30) Priority Data: PCT/EP94/03397 15 October 1994 (15.10.94) WO (34) Countries for which the regional or international application was filed: AT et al. 9422157.9 3 November 1994 (03.11.94) GB 9507523.0 11 April 1995 (11.04.95) GB			
(71) Applicant (for all designated States except US): DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only): YU, Shukun [CN/SE]; Gunnar Hejdemans Gata 29, S-212 40 Malmo (SE). BO-JSEN, Kirsten [DK/DK]; Hvidehusvej 13, DK-3450 Allerod (DK). MARCUSSEN, Jan [DK/DK]; Knabrostraede 25, 2, DK-1210 Copenhagen K (DK).			
(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).			
(54) Title: α -1,4-GLUCAN LYASE AND ITS USE IN THE PRODUCTION OF 1,5-ANHYDROFRUCTOSE			
(57) Abstract			
<p>An enzyme isolatable from algae is described. Also, a method of preparing the sugar 1,5-D-anhydrofructose is described. The method comprises treating an α-1,4-glucan with an α-1,4-glucan lyase wherein the enzyme is used in substantially pure form. In a preferred embodiment, if the glucan contains links other than and in addition to the α-1,4-links, the α-1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

α -1,4-glucan lyase and its use in the production of 1,5-anhydrofructose

5 The present invention relates to an enzyme α -1,4-glucan lyase ("GL") and, in particular, its use to prepare 1,5-D-anhydrofructose ("AF") from substrates based on α -1,4-glucan.

10 The present invention also relates to the use of a sugar, in particular 1,5-D-anhydrofructose ("AF") prepared by the method of the present invention, as an anti-oxidant, in particular as an anti-oxidant for food stuffs and beverages.

15 The present invention relates to the use of 1,5-D-anhydrofructose ("AF"), in particular AF prepared by the method of the present invention, as a sweetener, in particular as a sweetener for foodstuffs and beverages, preferably human foodstuffs and beverages.

20 FR-A-2617502 and Baute *et al* in *Phytochemistry* [1988] vol. 27 No.11 pp3401-3403 report on the production of AF in *Morchella vulgaris* by an apparent enzymatic reaction. The yield of production of AF is quite low. Despite a reference to a possible enzymatic reaction, neither of these two documents presents any amino acid sequence data for any enzyme let alone any nucleotide sequence information. These documents say that AF can be a precursor for the preparation of the antibiotic pyrone microthecin.

25 Yu *et al* in *Biochimica et Biophysica Acta* [1993] vol 1156 pp313-320 report on the preparation of GL from red seaweed and its use to degrade α -1,4-glucan to produce AF. The yield of production of AF is quite low. Despite a reference to the enzyme GL this document does not present any amino acid sequence data for that enzyme let alone any nucleotide sequence information coding for the same.

30 A typical α -1,4-glucan based substrate is starch. Today, starches have found wide uses in industry mainly because they are cheap raw materials.

Starch degrading enzymes can be grouped into various categories. The starch hydrolases produce glucose or glucose-oligomers. A second group of starch degrading enzymes are phosphorylases that produce glucose-1-phosphate from starch in the presence of inorganic phosphate.

5

AF has also been chemically synthesised - see the work of Lichtenthaler in Tetrahedron Letters Vol 21 pp 1429-1432. However, this chemical synthesis involves a large number of steps and does not yield large quantities of AF.

10

The chemical synthetic route for producing AF is therefore very expensive.

There is therefore a need for a process that can prepare AF in a cheap and easy manner and also in a way that enables large quantities of AF to be made.

15

Furthermore, anti-oxidants are typically used to prevent oxygen having any deleterious effect on a substance such as a foodstuff. Two commonly used anti-oxidants are GRINDOX 142 and GRINDOX 1029. These anti-oxidants contain many components and are quite expensive to make.

20

There is therefore a need to have a simpler and cheaper form of anti-oxidant.

Furthermore, sweeteners are often used in the preparation of foodstuffs and beverages. However, many sweeteners are expensive and complex to prepare.

25

There is therefore a need to have a simpler and cheaper form of sweetener.

According to a first aspect of the present invention there is provided an enzyme comprising at least any one of the amino acid sequences shown as SEQ. ID. No.s 3-4, or any variant thereof.

30

According to a second aspect of the present invention there is provided a nucleotide sequence coding for the enzyme of the first aspect of the present invention.

Preferably the nucleotide sequence is a DNA sequence.

According to a third aspect of the present invention there is provided a nucleotide sequence comprising a sequence that is the same as, or is complementary to, or has
5 substantial homology with, or contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

According to a fourth aspect of the present invention there is provided a method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with
10 the enzyme α -1,4-glucan lyase, characterised in that enzyme is used in substantially pure form and wherein the enzyme is isolated from algae alone.

According to a fifth aspect of the present invention there is provided a method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with
15 the enzyme α -1,4-glucan lyase characterised in that enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.

According to a sixth aspect of the present invention there is provided the sugar 1,5-D-anhydrofructose when prepared by the method of the present invention.
20

According to a seventh aspect of the present invention there is provided the use of a reagent that can increase the hydrophobicity of the reaction medium to increase the stability and activity of the GL enzyme.
25

According to an eighth aspect of the present invention there is provided the use of AF prepared by the method of the present invention as an anti-oxidant.

According to a ninth aspect of the present invention there is provided the use of AF prepared by the method of the present invention as a sweetener.
30

Preferably the enzyme is obtainable from algae, preferably it is obtainable from *Gracilariopsis lemaneiformis*.

5 Preferably the enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.

Preferably the enzyme is obtained from the expression of a nucleotide sequence coding for the enzyme.

10 Preferably the nucleotide sequence is a DNA sequence.

Preferably the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

15 Preferably if the glucan contains links other than and in addition to the α -1,4- links the α -1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.

20 Preferably the glucan is starch.

Preferably a glucanohydrolase is used in conjunction with the α -1,4-glucan lyase.

25 Preferably the starch is used in high concentration - such as up to about 25% solution.

Preferably the hydrolase is at least one of pullanase or isoamylase.

30 Preferably the α -1,4-glucan lyase is bound to a support or, more preferably, is in a dissolved form.

Preferably the enzyme is isolated and/or further purified from algae alone using a gel that is not degraded by the enzyme.

5 Preferably the gel is based on dextrin or derivatives thereof, preferably the gel is a cyclodextrin - more preferably β -cyclodextrin.

Preferably the substrate is treated with the enzyme in the presence of a buffer.

10 Alternatively, preferably the substrate is treated with the enzyme in the presence of at least substantially pure water.

Preferably the substrate is treated with the enzyme in the absence of a co-factor.

15 Preferably the enzyme is used in combination with amylopectin or dextrin.

20 The terms "variant" or "homologue" include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid or amino acid from or to the respective sequence providing the resultant sequence has the respective ability to code for or act as an enzyme according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as a promoter. With respect to sequence homology, preferably there is more than 80% homology, more preferably at least 85% homology, more preferably at least 90% homology, even more preferably at least 95% homology, more preferably at least 98% homology. Thus, the expression "substantial homology" covers homology with respect to structure and/or nucleotide components and/or biological activity.

30 The expression "contains any suitable codon substitutions" covers any codon replacement or substitution with another codon coding for the same amino acid or any addition or removal thereof providing the resultant enzyme has lyase activity.

In other words, the present invention also covers a modified DNA sequence in which at least one nucleotide has been deleted, substituted or modified or in which at least one additional nucleotide has been inserted so as to encode a polypeptide having the activity of a glucan lyase, preferably having an increased lyase activity.

5

AF prepared by the present method was confirmed and characterised by ^{13}C NMR.

One of key advantages of the present method is that the sugar 1,5-D-anhydrofructose can be prepared in much larger quantities than before and by a method that is relatively easier and cheaper than the known processes. For example the sugar can now be prepared in amounts of for example greater than 100g – such as 500g – compared to the prior art methods when only much smaller amounts were and could be produced – such as micro gram amounts.

15 Typical reactions that can be catalyzed by GL can be summarised as follows:

1). Amylopectin -----→ AF + limit dextrin

2). Amylose -----→ AF + limit dextrin

20

3). Dextrin -----→ AF + glucose

In reaction 1), the ratio of the two products depend on the structure of amylopectin or the distribution of α -1,6-glucosidic linkages in the amylopectin molecules.

25

In reaction 2) and 3), the ratio of the products depends on the degree of polymerisation (DP) number of the substrate. In reaction 3 the ratio between AF and glucose depends upon the DP. For example if the dextrin contains 10 glucose units the ratio AF:glucose would be 9:1.

30

Another advantage of the present invention is that glucans that contain links other than α -1,4- links can be substantially degraded – whereas before only partial degradation

was achieved. The substantial degradation of the 1,5-D-anhydrofructose precursor is one of the factors leading to the increased yields of 1,5-D-anhydrofructose.

Another advantage is that AF is a naturally occurring substance and therefore it has a potential for human purposes. For example, it can be converted to the antibiotic microthecin by AF dehydrase. Antibiotics are known for their uses in food bio-preservation, which is an important area in food technology. However, to date, the preparation of AF and also microthecin has had a number of disadvantages. For example, only small quantities could be produced. Also, the process was costly.

The present invention overcomes these problems by providing a larger production of and much cheaper production of AF and so also other products such as microthecin. In this regard, it is possible to prepare gram to kilogram amounts of AF.

A further advantage is that the lyase is stable for at least one year at 4°C and can be lyophilized without loss of activity.

Another advantage is that the lyase produces AF directly from starches and does not need the presence of any co-factors.

Another advantage is that the enzyme can be used in pure water. This result is very surprising.

Based on the simple properties of the present lyase, one can expect that the production cost of AF will be comparable to that of glucose. This is especially advantageous that the present lyase does not necessarily require the presence of any co-factors which are generally very expensive.

In general α -1,4-glucans can be used as substrate for the enzyme.

As a preferred substrate, starch is used.

In a preferred process, soluble or gelatinized starch or starch hydrolysate are used. The starch hydrolysates can be prepared either chemically or enzymatically.

5 If an enzyme is used for the partial starch degradation the enzyme can either be added before the addition of the lyase or any other additional starch degrading reagent (such as the enzyme glucanohydrolase) which may be added simultaneously.

10 The lyase will convert the glucan to AF. The enzyme will attack the substrate from the non reducing end and leave only the reducing sugar unconverted. The residual glucose can be removed by known methods some of which have been described here.

Using the reaction described here pure AF can be produced and also in large amounts.

15 Thus, in one embodiment, the α -1,4-glucan lyase is purified from algae - such as *Gracilariopsis lemaneiformis* - by affinity chromatography on β -cyclodextrin Sepharose, ion exchange chromatography on Mono Q HR 5/5 and gel filtration on Superose 12 columns. The purified enzyme produces 1,5-anhydro-D-fructose from α -1,4-glucans.

20 The enzymes of the present invention convert amylose and amylopectin to 1,5-anhydrofructose.

25 Among the maltosaccharides tested, we found that the lyase showed low activity towards maltose, and lower activity to maltotriose and maltoheptaose with the highest activity to maltotetraose and maltopentaose. The enzyme showed no substrate inhibition up to a concentration 10 mg ml⁻¹ among these maltosaccharides.

The enzymes from the preferred source have been sequenced and the amino acid sequences are presented later.

30

Also presented later are the DNA sequences coding for the enzymes.

The present invention therefore describes a new starch degrading enzyme – namely a new α -1,4-glucan lyase. This is an enzyme that has been purified and characterized for the first time.

5 As mentioned above, the present invention also relates to some specific uses of AF. In particular, the present invention relates to the use of 1,5-D-anhydrofructose ("AF"), as an anti-oxidant, in particular as an anti-oxidant for food stuffs and beverages.

10 Therefore according to the present invention there is provided the use of 1,5-D-anhydrofructose (AF) as an anti-oxidant.

Preferably AF is or is used in an edible substance.

15 Preferably AF is used in or as a foodstuff or beverage.

Preferably, AF is used in combination with another anti-oxidant.

Preferably the AF is prepared by the method according to the present invention.

20 The main advantages of using AF as an anti-oxidant are that it is a natural product, it is likely that it is non-metabolisable at least for humans, it is easy to manufacture, it is water-soluble, and it is generally non-toxic.

25 A preferred embodiment the present invention therefore relates to the enzymatic preparation of pure AF which can be used as an attractive water soluble antioxidant for food and non-food purposes. Examples are given below for the use of AF as an antioxidant in food formulations.

30 Experiments showed that the AF is comparable with known high quality commercial available food antioxidants.

Non-food examples include use in polymer chemistry as oxygen scavengers during the synthesis of polymers.

Also, AF could be used for the synthesis of bio-degradable plastic.

5

Experiments have shown that AF can be an efficient reducing agent (antioxidant), as it can easily reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid.

10

AF is a naturally occurring substance and therefore it has a tremendous potential for use as an acceptable antioxidant. AF can also be converted into the antibiotic microthecin by AF dehydrase. Antibiotics are known for their uses in food biopreservation, an important area in food biotechnology.

15

In another aspect, the present invention also relates to the use of 1,5-D-anhydrofructose as a sweetener, in particular as a sweetener for foodstuffs and beverages, preferably human foodstuffs and beverages.

20

Thus according to this aspect of the present invention there is provided the use of 1,5-D-anhydrofructose as a sweetener.

Preferably the AF is used as or in a human foodstuff or beverage.

25

The AF may be used in any desired amount such as a 5% solution or 100mg/kg to 500 mg/kg.

The advantages of using AF as a sweetener are that it is a natural product, it is generally non-toxic, it is water soluble, it is likely that it is non-metabolisable at least for humans, and it is easy to manufacture.

30

The present invention therefore also relates to a novel application of AF as a sweetener.

Preferably the AF is prepared by the method according to the present invention.

Further aspects of the present invention include:

5 a method of preparing the enzyme α -1,4-glucan lyase (GL) comprising isolating the enzyme from algae alone;

 a nucleotide sequence coding for the enzyme α -1,4-glucan lyase, preferably wherein the sequence is not in its natural environment (i.e. it does not form part of the
10 natural genome of a cellular organism capable of expressing the enzyme, preferably wherein the nucleotide sequence is a DNA sequence;

 the use of β -cyclodextrin to purify an enzyme, preferably GL.

15 Other preferred embodiments of the present invention include any one of the following: A transformed cell, tissue, organ or host organism having the capability of producing AF as a consequence of the introduction of a DNA sequence as herein described; such a transformed host organism which is a microorganism - preferably wherein the host organism is selected from the group consisting of bacteria, moulds,
20 fungi and yeast; preferably the cell, tissue, organ or host organism is obtained from or is any one of the group consisting of *Saccharomyces*, *Kluyveromyces*, *Aspergillus*, *Trichoderma Hansenula*, *Pichia*, *Bacillus Streptomyces*, *Eschericia* such as *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *bacillus subtilis*, *Bacillus amyloliquefascien*, *Eschericia coli*.; A method for preparing the sugar 1,5-D-anhydrofructose comprising
25 the use of a transformed host organism expressing a nucleotide sequence encoding the enzyme α -1,4-glucan lyase, preferably wherein the nucleotide sequence is a DNA sequence, preferably wherein the DNA sequence is one of the sequences hereinbefore described; A vector incorporating a nucleotide sequence as hereinbefore described, preferably wherein the vector is a replication vector, preferably wherein the vector is
30 an expression vector containing the nucleotide sequence downstream from a promoter sequence, preferably the vector includes a marker (such as a resistance marker); Cellular organisms, or cell line, transformed with such a vector; A method of

producing the product α -1,4-glucan lyase or any nucleotide sequence or part thereof coding for same, which comprises culturing such an organism (or cells from a cell line) transfected with such a vector and recovering the product.

- 5 In particular, in the expression systems, the enzyme should preferably be secreted to ease its purification. To do so the DNA encoding the mature enzyme is fused to a signal sequence, a promoter and a terminator from the chosen host.

10 For expression in *Aspergillus niger* the *gpdA* (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused to the 5' end of the DNA encoding the mature lyase. The terminator sequence from the *A. niger trpC* gene is placed 3' to the gene (Punt, P.J. *et al* 1991 - (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker for *A. niger*.
15 Examples of selection markers for *A. niger* are the *amdS* gene, the *argB* gene, the *pyrG* gene, the *hygB* gene, the *BmlR* gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants. Eventually the construction could be transformed into a protease deficient strain to reduce the
20 proteolytic degradation of the lyase in the culture medium (Archer D.B. *et al* 1992 - Biotechnol. Lett. 14, 357-362).

Instead of *Aspergillus niger* as a host, other industrial important microorganisms for which good expression systems are known could be used such as: *Aspergillus oryzae*,
25 *Aspergillus sp.*, *Trichoderma sp.*, *Saccharomyces cerevisiae*, *Kluyveromyces sp.*, *Hansenula sp.*, *Pichia sp.*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Bacillus sp.*, *Streptomyces sp.* or *E. coli*. Also, other forms of algae may be used.

The following sample was accepted as a deposit in accordance with the Budapest Treaty at the recognised depositary The Culture Collection of Algae and Protozoa (CCAP) at Dunstaffnage Marine Laboratory PO Box 3, Oban, Argyll, Scotland, United Kingdom, PA34 4AD on 11 October 1994:

5

Gracilariopsis lemaneiformis (CCAP 1373/2) - [ref. GLSC-1 (California)].

Thus a highly preferred embodiment of the present invention includes a GL enzyme or a nucleotide sequence coding for same obtainable from the algae that is the subject of deposit CCAP 1373/2.

10

The present invention will now be described only by way of example, in which reference shall be made to Figures which shows the results of some electrophoretic studies on algal α -1,4-glucan lyases, and Figure 2 which shows the results of some isoelectric focus studies on α -1,4-glucan lyases on a gel with a pH gradient of pH 3 to pH 9. Figures 1 and 2 are described in more detail later.

15

PREPARATION OF α -1,4-GLUCAN LYASE

The enzyme α -1,4-glucan lyase according to the present invention (e.g. for use in preparing AF) may be isolated from algae alone, preferably *Gracilariopsis lemaneiformis*, more preferably *Gracilariopsis lemaneiformis* from Santa Cruz, (California).

20

The initial enzyme purification can be performed by the method as described by Yu *et al* (ibid). However, preferably, the initial enzyme purification includes an optimized procedure in which a solid support is used that does not decompose under the purification step. This gel support further has the advantage that it is compatible with standard laboratory protein purification equipment. The details of this optimized purification strategy are given later on. The purification is terminated by known standard techniques for protein purification. The purity of the enzyme can be readily established using complementary electrophoretic techniques.

25

30

CHARACTERISATION OF THE ENZYME

Amino acid sequence analysis

5 The α -1,4-glucan lyase from *Gracilariopsis lemaneiformis* was digested with either
endoproteinase Arg-C from *Clostridium histolyticum* or endoproteinase Lys-C from
Lysobacter enzymogenes, both sequencing grade purchased from Boehringer
Mannheim, Germany. For digestion with endoproteinase Arg-C, freeze dried lyase
10 (0.1 mg) was dissolved in 50 μ l 10 M urea, 50 mM methylamine, 0.1 M Tris-HCl,
pH 7.6. After overlay with N₂ and addition of 10 μ l of 50 mM DTT and 5 mM EDTA
the protein was denatured and reduced for 10 min at 50°C under N₂. Subsequently, 1
 μ g of endoproteinase Arg-C in 10 μ l of 50 mM Tris-HCl, pH 8.0 was added, N₂ was
overlayed and the digestion was carried out for 6h at 37°C. For subsequent cysteine
derivatization, 12.5 μ l 100 mM iodoacetamide was added and the solution was
15 incubated for 15 min at RT in the dark under N₂.

For digestion with endoproteinase Lys-C, freeze dried lyase (0.1 mg) was dissolved
in 50 μ l of 8 M urea, 0.4 M NH₄HCO₃, pH 8.4. After overlay with N₂ and addition
of 5 μ l of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C
20 under N₂. After cooling to RT, 5 μ l of 100 mM iodoacetamide was added for the
cysteines to be derivatized for 15 min at RT in the dark under N₂.

Subsequently, 90 μ l of water and 5 μ g of endoproteinase Lys-C in 50 μ l of 50 mM
tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for
25 24h at 37°C under N₂.

The resulting peptides were separated by reversed phase HPLC on a VYDAC C18
column (0.46 x 15 cm; 10 μ m; The Separations Group; California) using solvent A:
0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were
30 rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3 μ m; Dr. Ole Schou,
Nov Nordisk, Denmark) using the same solvent system prior to sequencing on an
Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

The amino acid sequence information from the enzyme derived from *Gracilariopsis lemaneiformis* is shown below (corresponds to SEQ. ID. No. 3) where the sequenced peptides are underlined.

```

5  MFPTLTFIAP SALAASTFVG ADIRSGIRIQ SALPAVRNAV RRSKHYNVSM TALSDKQTAI
   SIGPDNPDGI NYQNYDYIPV AQETPLSNTN WYAAGSSTPG GITDWTATMN VKFDRIDNPS
   YSNNHPVQIQ VTSYNNNSFR IRFNPDGPIR DVSRGPILKQ QLTWIRNOEL AQGCNPNMSF
   SPEGFLSFET KDLNVIIYGN CKMRVTKKDG YLVMENDECN SQSDGNKCRG LMYVDRLYGN
   AIASVQTNFH KDTSRNEKFY GAGEVNCRYE EQGKAPTYVL ERSGLAMTNY NYDNLNYNQP
10 DVVPPGYPDH PNYIIPMYA APWLVVQGA GTSKQYSYGW FMDNVSQSYM NTGDTAWNCG
   QENLAYMGAQ YGPFQHFVY GDGDGLEDVV KAFSFLQGKE FEDKKLNKRS VMPPKYVFGF
   FQGFGALSL LKQNLPA GEN NISVQEIVEG YQDNDYPFEG LAVDVDMQDD LRVFTTKPEY
   WSANMVGEGG DPNNRSVFEW AHDRGLVCQT NVTCLFRNDN SGKPYEVNQT LREKQLYTKN
   DSLNTTDEGT TSDGPGDAYI GHLDYGGGVE CDAIFPDWGR PDVAQWWGEN YKKLESIGLD
15 FVWQDMTVPA MPMHRLGDAV NKNSGSSAPG WPNENDPSNG RYNWKSYPHQ VLVTDMRYGA
   EYGREPMVSQ RNIHAYTLCE STRREGIVGN ADSLTKFRRS YIISRGGYIG NOHFGGMWVG
   DNSATESYLQ MMLANLINMN MSCLPLVGS D IGGFTQYNDV GDPTPEDLMV RFVQAGCLLP
   WFRNHYDRWI ESKKHGKKYQ ELYMYPGQKD TLKKFVEFRY RWQEVLYTAM YQNATTGEPI
   IKAAPMYNND VNVYKSONDH FLGGHDDGYR ILCAPVVREN ATSREVYLPV YSKWFEKGP
20 EDTKPLENEI OGGQTLNYA APLNDSPIFV REGTILPTRY TLDGVNKSIN TYTDNDPLVF
   ELFPLENNQA HGLFYHDDGG VTTNAEDFGK YSVISVCAAQ EGSQMSVKFD NEVYEHQWGA
   SFYVRVRNMG APSNINVSSQ IGQQDMQQSS VSSRAQMFTS ANDGEYWVDQ STNSLWLKLP
   GAVIQDAAIT VR

```

25 In addition, for the characterization of α -1,4-glucan lyase from a red seaweed *Gracilariopsis lemaneiformis* collected in Santa Cruz, California and apparently not infected by fungi, the following amino acid composition of the lyase was found:

16

	Amino acid residues	mol % of each residue

	Asx	15.42
	Thr	5.24
5	Ser	6.85
	Glx	9.46
	Pro	5.46
	Gly	9.08
	Ala	5.38
10	1/2Cys	1.57
	Val	6.60
	Met	2.90
	Ile	3.66
	Leu	6.00
15	Tyr	6.00
	Phe	4.37
	His	1.65
	Lys	4.44
	Arg	4.17
20	Trp	1.75
	Total:	100.00

25 Comparing the peptide sequences from the Californian algae with the amino acid sequence from a fungally infected algae from China (described in co-pending PCT patent application No. PCT/EP/0339) showed a high degree of homology (72-79% identity between the amino acid sequence in SEQ. ID. No. 3 and No. 4 generated from the PCR fragments and the corresponding sequences in the GL obtained from the algae from China) between the protein sequences.

30 The alignment of the protein sequences showed that sequence position 1 to 50 of SEQ. ID. No. 3 represents a signal sequence and sequence position 51 to 1092 represents the mature protein.

SEQ. I.D. No.3 has the following characteristics:

Number of residues:1092

Molecular weight (MW): 123169

5 Amino acid composition (including the signal sequence):

64 Ala	14 Cys	18 His	33 Met
56 Thr	48 Arg	55 Gln	45 Ile
49 Phe	22 Trp	89 Asn	49 Glu
65 Leu	59 Pro	67 Tyr	73 Asp
10 94 Gly	46 Lys	73 Ser	73 Val

SEQ. ID. No. 4 is a partial sequence corresponding to amino acid 287 to 861 in SEQ. ID. No. 3. The identity between the two sequences is 81%.

15 DNA sequence analysis

DNA from *Gracilariopsis lemaneiformis* was isolated as described by Saunders (1993) with the following modification: The polysaccharides were removed from the DNA by ELUTIP-d (Schleicher & Schuell) purification instead of gel purification. (Ref: 20 Saunders, G.W. (1993). Gel purification of red algal genomic DNA: An inexpensive and rapid method for the isolation of PCR-friendly DNA. Journal of phycology 29(2): 251-254 and Schleicher & Schuell: ELUTIP-d. Rapid Method for Purification and Concentration of DNA.)

25 For PCR, the appropriate DNA molecule was prepared by use of the Gene Amp DNA Amplification Kit (Perkin Elmer Cetus, USA) and in accordance with the manufactures instructions except that the Taq polymerase was added later (see PCR cycles) and the temperature cycling was changed to the following:

18

PCR cycles:

no of cycles	C	time (min.)
1	98	5
	60	5
5	addition of Taq polymerase and oil	
	35	94
		1
		47
		2
		72
		3
	1	72
		20
10		

PCR fragments were cloned into pT7Blue (from Novagen) or ScriptTMSK(+) following the instructions of the supplier.

The double stranded DNA was sequenced essentially according to the dideoxy method of Sanger *et al.* (1979) using the Auto Read Sequencing Kit (Pharmacia) and the Pharmacia LKB A.L.F.DNA sequencer. (Ref: Sanger, F., Nicklen, S. and Coulson, A.R.(1979). DNA sequencing with chain-determinating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.). The sequences are shown as SEQ. ID. No.s 1-2.

For the initial PCR, the following three oligonucleotide sequences were generated from two peptide sequences from the Californian algae to generate a PCR fragment of app. 970 bp.

Primer 1: ATGAC(GATC)AA(CT)TA(CT)AA(CT)TA(CT)GA(CT)AA

Primer 2: (AG)TG(GATC)GGCATCAT(GATC)GC(GATC)GG(GATC)AC

Primer 3: GTCAT(GA)TC(CT)TGCCA(GATC)AC(GA)AA(GA)TC

Primer 1 corresponds to the codons for amino acids 287-293 in SEQ. I.D. No. 3.

Primer 2 and primer 3 correspond to codons (of the complementary strands) for amino acids 608-614 and 599-607 of SEQ. ID. No. 3, respectively.

Primer 1 was used as the upstream primer and primer 2 was used as the downstream primer in the first PCR amplification. In the second PCR amplification primer 1 was used as the upstream primer and primer 3 was used as the downstream primer. A PCR fragment of the expected size was generated and cloned into the pT7blue vector from Novagen. Two independent plasmids containing a PCR fragment were sequenced and it was seen that those two cloned PCR fragments contained the codons for peptide sequences originating from two different proteins. This indicates that there are at least two different genes coding for α -1,4-glucan lyase in the Californian algae containing sequences shown as SEQ. ID. No.s 1-2 - their putative amino acid sequences are shown as SEQ. ID. No.s 3-4.

Further PCR using the Pfu polymerase from Stratagene were made according to the manufactures instruction using the same temperature cycling as for the Taq polymerase. The PCR fragments were cloned into either the pT7Blue plasmid from Novagen or the pCR-Script™ SK(+) plasmid from Stratagene.

Four additional PCR amplifications were performed using the following primers.

For the first PCR the primer

GA(CT) AA(CT) CC(GATC) GA(CT) GG(GATC) AT(ATC) (GA)A(CT) TA

corresponding to codons for amino acids 65-72 of SEQ. ID. No.3 was used as the upstream primer and the primer

(GA)GA (TG)AC ATT (GA)TC CA(AT) AAA CCA

generated from the initial PCR corresponding to codons (of the complementary strand) for amino acids 340-346 of SEQ. ID. No.3 was used as the downstream primer.

In the second PCR the primer

GT(GA) GAT GT(GT) GAT ATG CAA (GC)A(AT) GA

- 5 generated from the initial PCR corresponding to codons for amino acids 463–470 of SEQ. ID. No.3 was used as the upstream primer and the primer

CCA CAT (GATC)CC (GATC)CC (GA)AA (GA)TG (CT)TG (GA)TT

- 10 corresponding to codons (of the complementary strand) for amino acids 711–718 of SEQ. ID. No.3 was used as the downstream primer.

In the third PCR the primer

- 15 GTG AGT CTA CTA GGA GGG AA

generated from the initial PCR corresponding to codons for amino acids 389–395 of SEQ. I.D.No.4 was used as the upstream primer and the primer

- 20 A(GA) (GA)AA (GA)TG (GA)TC (GA)TT (CT)TG

corresponding to codons (of the complementary strand) for amino acids 566–571 of SEQ. ID. No. 4 was used as the downstream primer.

- 25 In the fourth PCR the primer

TTC CCA GA(CT) TGG GGT CGA CC

- 30 generated from the initial PCR corresponding to codons for amino acids 575–581 of SEQ. ID. No. 3 was used as the upstream primer and the primer

GT(GA) AA(GA) TC(GATC) GG(GATC) CC(GA) AA

corresponding to codons (of the complementary strand) for amino acids 897-902 of SEQ. ID. No. 3 was used as the downstream primer.

5 The first PCR amplification generated a DNA sequence corresponding to nucleotides 193-1038 in SEQ. ID. No. 1.

The second PCR amplification generated DNA sequences corresponding to nucleotides 1387-2154 in SEQ. ID. No. 1 and nucleotides 526-1284 in SEQ. ID. No. 2.

10 The third PCR amplification generated a DNA sequence corresponding to nucleotides 1165-1712 in SEQ. ID. No. 2.

The fourth PCR amplification generated a DNA sequence corresponding to nucleotides 1723-2706 in SEQ. ID. No. 1.

15 To obtain the 5' and 3' end of SEQ. ID. No.3 the RACE (rapid amplification of cDNA ends) procedure was performed (Michael, A.F., Michael, K.D. & Martin, G.R.(1988). Proc.Natl.Acad.Sci.USA 85:8998-9002) using the 5'Race system from Gibco BRL for the 5'Race. Total RNA was isolated according to Collinge *et al.*(Collinge, D.B.,
20 Milligan, D.E., Dow, J.M., Scofield, G. & Daniels, M.J.(1987). Plant Mol Biol 8:405-414). The 5'Race was done according to the protocol of the manufacturer, using 1µg of total RNA and the sequence

25 GGT AGC GGT CCA GTC GGT GAT GCC

(identical to the complementary strand of nucleotides 301-324 in SEQ. ID. No. 1) as a primer in the first strand synthesis.

30 The sequence

AGA GCC GGC AGC ATA CCA GTT GGT GTT GG

identical to the complementary strand of nucleotides 260–288 in SEQ. ID. No. 1 was used for the PCR amplification.

In the 3'Race the following primer

5

GAA GGA TCC GTC GAC ATC GAT AAT ACG ACT GAA TTC GGG ATT TTT
TTT TTT TTT TTT

was used for the first strand synthesis.

10

The gene specific primer

GAC GGC TAT CGT ATT CTC TGC

15

identical to nucleotides 2599–2619 in SEQ. ID. No. 1 was used in the first PCR amplification, while primer

TAC CTG CCT GTG TAT AGC AAG

20

corresponding to nucleotides 2659–2679 was used as the gene specific primer in the second PCR amplification.

The sequence

25

AAG GAT CCG TCG ACA TCG ATA AT

was used as the downstream primer in both PCR amplifications. The GeneAmp RNA PCR kit from Perkin Elmer was used for the 3'Race.

30

After the 5' and 3' Race the PCR fragments were blunt ended with the Pfu polishing kit from Stratagene before cloning into the pCR Script™ SK(+) plasmid.

BIOCHEMICAL CHARACTERIZATION

Electrophoretic properties of the α -1,4-glucan lyase purified from the red alga *Gracilariopsis lemaneiformis* collected in Santa Cruz, Californian (USA), as compared with
 5 lyases collected in Qingdao (China) and Sucre Pennisula (Venezuela), and with lyases isolated from the fungi *Morchella costata*, *M. vulgaris*.

Two lyases (GLsc1-lyase and GLsc2-lyase) were purified from the red alga
Gracilariopsis lemaneiformis collected in Santa Cruz, California (USA), using the
 10 procedure developed for the purification of the lyase from the red alga collected from Qingdao China. The two forms were purified to electrophoretic homogeneity (Figure 1A and 1B). Partial amino acid sequences were obtained from the purified GLsc1-lyase and were used to generate PCR primers. The molecular mass was estimated to
 15 116 kDa for both GLsc1-lyase and GLsc2-lyase, by the SDS-PAGE method (Figure 1A).

On the native PAGE (Figure 1B), GLsc1-lyase and GLsc2-lyase migrated slower than GL1-lyase. On the isoelectrofocusing gel, GLsc1-lyase showed a pI of 4.1 (Fig. 2). A comparison of the relative migration rates and the pI values of the different lyases
 20 studied are listed in the following table.

Enzyme name	Origin	Migration	pI
GL1-lyase	QC	++++	3.8
GLsc1-lyase	SCC	+++	4.1
GLsc2-lyase	SCC	+++	4.1
GLv-lyase	SpV	+++	4.1
MV-lyase	MvA	++	4.4
MC-lyase	MCA	+	4.5

CODES:

QC = Qingdao, China

SCC = Santa Cruz, California

SpV = Sucre peninsula, Venezuela

5 MvA = *Morchella vulgaris*, ATCC

McA = *Morchella costata*, ATCC

10 The relative migration rates were estimated from native PAGE on gels with 8–25% gradient; the pI values were estimated by isoelectrofocusing on gels with a pH gradient of 3–9. From the table it is seen that the GLq1-lyase exhibited the fastest migration rate on native-PAGE and exhibited the lowest pI values of 3.8 while the MC-lyase showed the slowest migration rate and the highest pI value of 4.5.

15 The Figures are now discussed in more detail.

Figure 1. Electrophoresis of algal α -1,4-glucan lyases.

A. SDS-PAGE on 8–25% gradient gels. The gel was stained with PhastGel Blue R.

20 Lane 1: the molecular markers of phosphorylase b (97.4kDa); bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and β -lactalbumin (14.4 kDa).

25 Lane 2: the purified GLq1-lyase from the red alga *Gracilariopsis lemaneiformis* collected in Qingdao (China).

Lanes 3 and 5: the purified GLsc1-lyase from the red alga *Gracilariopsis lemaneiformis* collected in Santa Cruz, California (USA).

30 Lane 4: the purified GLsc2-lyase from the red alga *Gracilariopsis lemaneiformis* collected in Santa Cruz, California (USA).

B. Native-PAGE on 8-25% gradient gel. The gel was stained with PhastGel Blue R.

Lane 1 and 4: the purified GLq1-lyase from the red alga *Gracilariopsis lemaneiformis* collected in Qingdao (China).

5

Lane 2: the purified GLsc2-lyase from the red alga *Gracilariopsis lemaneiformis* collected in Santa Cruz, California (USA).

10

Lane 3: the purified GLsc1-lyase from the red alga *Gracilariopsis lemaneiformis* collected in Santa Cruz, California (USA).

Lane 5: the protein markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

15

Figure 2. Isoelectric focusing of the α -1,4-glucan lyases on a gel with a pH gradient of pH 3 to pH 9. The gel was stained with PhastGel Blue R.

Lane 1: GLsc1-lyase purified from *Gracilariopsis lemaneiformis* collected in Santa Cruz California showing a pI of 4.1.

20

Lane 2: GLq1-lyase purified from *G. lemaneiformis* collected in Qingdao (China) showing a pI of 3.8.

25

Lane 3: the pI markers: 1 amyloglucosidase (3.50), 2 soybean trypsin inhibitor (4.55), 3 β -lactoglobulin A (5.20), 4 bovine carbonic anhydrase B (5.85), 5 human carbonic anhydrase B (6.55), 6 horse myoglobin (acidic, 6.85), 7 horse myoglobin (basic 7.35), 8 lentil lectin (acidic 8.15), 9 lentil lectin (middle, 8.45), 10 lentil lectin (baic, 8.65).

30

Further studies showed that K_m was 3.76 mg/ml for amylopectin and 3.37 mg/ml for glycogen.

DEGRADATION STUDIES

The degradation rates of the lyase on various substrates are given below.

	Substrate	AF released (nmol)
5	Maltose	657
	Maltotriose	654
10	Maltotetraose	670
	Maltopentaose	674
	Maltohexaose	826
15	Maltoheptaose	865
	Dextrin 20	775
20	Dextrin 15	775
	Dextrin 10	844
	Amylopectin	732
25	Glycogen	592

30 Reaction conditions: The reaction mixture contained 10 mM of HOAc-NaOAc (pH 3.8). The substrate concentration was 10 mg/ml. The final volume was 100 ul after the addition of lyase and water. The reaction time was 40 min at 45°C.

35 The lyase was not able to degrade pullulan, nigeran tetrasaccharide, trehalose, isomaltose, glucose, α -, β - and γ -cyclodextrins. The lyase degraded panose and nigerose though at a slow rate.

40 The temperature optimum for the lyase was 48°C when amylopectin was used as substrate and 50°C when glycogen was used as substrate. At 50°C, the reactivity of glycogen was similar to that of amylopectin; below 50°C, amylopectin was a better substrate than glycogen.

The pH optimum range for the lyase was between pH 3.5 and pH 7.0; the optimal pH was 3.8. The buffers used in the pH tests were glycine-HCl (pH 2.2-3.6); NaOAc-HOAc (pH 3.5-5.5); Mes-NaOH (pH 5.5-6.7); Mops-NaOH (pH 6.0-8.0) and bicine-NaOH (pH 7.6-9.0). All buffers used were 40 mM.

5

At a final concentration of 2 mM, p-chloromercuribenzoic acid (PCMB) inhibited the lyase activity by 96%, indicating the -SH group(s) is essential for the enzymatic activity.

10

In immunological tests of the lyase by Western blotting, the results showed that the antibodies to the algal lyase could recognize the fungal lyase (of our earlier PCT patent application PCT/EP94/03397) both in cell-free extracts and in purified form, as revealed by Western blottings. The antibodies to the algal lyase purified from the algae collected in China (of our earlier PCT patent application PCT/EP94/03397) also

15

recognized the lyase from the algae collected in Santa Cruz, California.

Further aspects and experiments relating to the enzyme according to the present invention will now be described.

20

THE SOLUBLE ENZYME SYSTEM

1. Effect of pH on the stability and activity of the lyase isolated from *Gracilariopsis lemaneiformis*.

25

Two buffer systems, namely HOAc and NaOAc and sodium citrate - citric acid in a concentration of 5 mM - were tested at 37°C. The pH range tested was from pH 3 to pH 5.2. The lyase showed maximum activity in a pH range between 3.6 to 4.2. At pH 3, the stability and activity of the enzyme decreased by about 90%. At pH 5.2, the activity decreased by about 64%. However, the enzyme was considerably more stable

30

at this pH than at pH 3, as the AF yield obtained at pH 5.2 was 75% of the AF yield obtained at pH 3.8. Slightly higher AF yield was obtained in the HOAc and NaOAc buffer than in citrate buffer. This is not due to any differential effect of the two

buffers (final conc. is 125 μ M in the AF assay mixture) in the AF assay method.

2. Effect of temperature on the activity and stability of the lyase.

5 This experiment was conducted at optimal pH range. At 25°C the production of AF was linear up to at least 9 days. This indicates that no loss of activity and stability of the lyase occurred within 9 days. With increasing temperature, the stability of the enzyme decreased.

10 The half life of the enzyme activity at the following temperature was:

	30°C	5 days
	37°C	2.5 days
	40°C	less than 1 day
15	50°C	less than 1 day

3. Effect of substrate concentration on the stability of the lyase and AF yield.

20 It was observed that amylopectin and dextrans have a stabilizing effect on the lyase while the smallest substrate maltose does not. This was verified for both the soluble enzyme system and the immobilized enzyme system.

25 AF yield increases with the increase in amylopectin concentration up to 25%. In the case of dextrin, the AF yield decreases when the concentration exceeds 30% (30%, 40% and 50% were tested).

4. Activation and inactivation of lyase

30 No metal ions were found to be necessary for the activity and the enzyme catalysed reaction can surprisingly proceed in pure water. The fact that the addition of EDTA in the reaction mixture up to 20 mM had little effect on the activity clearly demonstrates that metal ions are not essential for the activity of the lyase enzyme

according to the present invention.

This means that in the AF purification step, the ion exchange chromatography step that takes away salts from the reaction system can be omitted, if water is used as reaction medium. However, inclusion of NaCl in the reaction mixture in a
5 concentration of 0.85% (0.145 M) can increase the AF yield up to 1-fold.

5. Substrate Specificity

10 Upon cooling solubilized starch will tend to form rigid gels when the starch concentration becomes too high. Therefore it is an advantage to utilize partly degraded starch as substrate for the 1,4-glucan lyase.

The specificity of α -1,4-glucan lyase for different oligosaccharides was tested. The
15 oligosaccharides were maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7). The oligosaccharides were dissolved in H₂O at a concentration of 8 mg/ml. The enzyme assay contained 150 μ l substrate G2/G3/G4/G5/G6/G7, 120 μ l 0.1M MES pH 6.3 and 30 μ l purified enzyme. The reaction mixture was incubated for 60 min at 30°C. Afterwards the reaction was
20 stopped by boiling for 3 min and 900 μ l absolute ethanol was added for precipitation. After centrifugation at 20.000 x g for 5 min at 4°C the supernatant was transferred to a new eppendorf tube and lyophilized.

The freeze-dried samples were dissolved in 1000 μ l H₂O and were filtrated through
25 a 0.22 μ m Millipore filter before 25 μ l of the sample was loaded on the Dionex HPLC.

6. HPLC

30 Analyses were performed on a Dionex 4500i chromatography system consisting of a GPM-2 pump and a PED detector which was used in pulse-amperometric detection mode. The anion exchange columns were a CarboPac PA-100 (4 x 250 mm) and a

30

CarboPac PA-100 guard column (3 x 25 mm) from Dionex. The eluent were 200 mM sodium hydroxide (A), 500 mM sodium acetate (B) and 18 M ohm de-ionized water (C). The pump was programmed in 2 different ways, method no. 1 and method no. 2:

5

Method no. 1:

10

Time, min	0.0	3.0	3.1	26.9	29.0
% A	10	10	50	50	10
% B	0	0	0	32	0
% C	90	90	50	18	90

Method no. 2:

15

Time, min.	0.0	30
% A	10	10
% B	0	0
% C	90	90

Standards:

20

Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose (all from Sigma) and 1,5-anhydrofructose were used as standards. All compounds were dissolved in 18 M ohm de-ionized water which was filtered through a 0.22 μ m Millipore filter before use.

7. Results

25

The analyses show that the purified enzyme was able to use maltooligosaccharides as substrate 1 for 1,5-anhydrofructose formation. When maltose was used as substrate, almost no 1,5-anhydrofructose was formed but when the other maltooligosaccharides

(G3-G7) were used, high amounts of this compound were produced. It is clear that higher amounts of 1,5-anhydrofructose were obtained when a longer maltooligosaccharide was used. This observation corresponds perfectly well with the theory of the lyase forming 1,5-anhydrofructose from the non-reducing end of the substrate, leaving only the terminal glucose molecule unchanged.

8. Formation of AF

α -1,4-glucan lyase according to the present invention hydrolyses starch to the end-product 1,5-anhydrofructose. The end-product was shown by HPLC, method 2. The enzyme assay contained 500 μ l amylopectin (20 mg/ml, dissolved in H₂O), 400 μ l 0.1 M MES pH 6.3 and 100 μ l purified enzyme. The reaction mixture was incubated at 30°C and the reaction was stopped by boiling after 30 or 120 min incubation. High-molecular oligosaccharides were precipitated by addition of 3 vol abs. ethanol and the sample was centrifuged and freeze-dried as described above. The samples were dissolved in 125 μ l H₂O and 25 μ l were applied on the HPLC column.

The HPLC elution profile clearly shows that α -1,4-glucan lyase produces 1,5-anhydrofructose by hydrolysis of starch. Equal amounts of 1,5-anhydrofructose were found after 30 and 120 min. incubation which indicate that the enzyme activity is not inhibited by the end-product 1,5-anhydrofructose.

¹³C NMR spectra (water) of AF prepared in this way shows that it adopts one major form giving rise to the following signals: δ 93.5 (quart, C-2), 81.5 (CH, C-5), 77.7 (CH, C-3), 72.6 (CH₂, C-1), 69.8 (CH, C-4), 62.0 (CH₂, C-6). Assignments are based on H-H C-H and C-H 2D correlation spectra.

9. The cooperative effect of lyase with pullulanase and isoamylase.

As it can be seen from the Table below, the inclusion of pullulanase in the reaction mixture will obviously increase the AF yield by about 15-23%, depending on whether soluble starch or amylopectin is used as substrate.

The cooperation of pullulanase and lyase in the production of AF.

Substrate	Lyase	Pullulanase	AF Yield (%)	Glc Yield (%)
Solubl.				
Starch	+	-	51	0
	-	+	0	0.37
	+	+	66.0	3.9
Amylo	+	-	48.0	0
-pectin	-	+	0	0.33
	+	+	71.3	3.7

+, enzyme added, - enzyme omitted.

The reaction mixture contained 0.3 ml 2% potato amylopectin (Sigma) in water or 0.3 ml 2% soluble starch (Merck), 2 μ l lyase and 0.36 units pullulanase (BM) as indicated.

5

The reaction was carried out at 30°C for 1 day. At the end of the reaction, samples were taken for AF and Glc analysis.

10

In the case of isoamylase, the advantage is that the optimal pH of the lyase overlaps with that of Pseudomonas isoamylase (pH 3.0-4.5). The problem, however, is that isoamylase will produce an excess amount of long chain amylose that precipitates from the solution, and therefore is no longer suitable as a substrate for the lyase. It can be expected that the cooperation of the lyase with isoamylase will be efficient, if the chain of amylose is not too long.

15

THE IMMOBILIZED ENZYME SYSTEM

Immobilization of the lyase was achieved by using succinimide-activated Sepharose (Affigel 15 gel, Bio-Rad) and glutaraldehyde-activated Silica gel (BM). The recovery of lyase activity after immobilization on Affigel 15 gel was between 40% to 50%. There may be some lyase that is still active after immobilization, but is inaccessible to the substrate because of the steric hindrance, especially in the case of macromolecules like starches. Immobilized enzymes used in the industry usually have an activity recovery of around 50%.

The most interesting thing of the Affigel 15 gel immobilized lyase is that its stability has been greatly improved at pH 5.5. When the column was operated at this pH, the stability was at least 16 days long. The pH shift in the stability is very important considering the optimal pH of pullulanase which is around pH 5.5. This is the prerequisite for the lyase and pullulanase to cooperate efficiently in the same reactor with the same physico-chemical environment. The soluble lyase has an optimal pH between 3.6 and 4.2, and at this pH range pullulanase shows little or no activity.

With the silica gel immobilized lyase, the activity recovery is very high, around 80-100%. However, the silica gel immobilized enzyme was not stable when the column was operated neither at pH 3.8 nor pH 5.5. It is possible that some lyase was adsorbed on the surface of the silica gel beads and was slowly released from the silica gel after each washing of the column. It may therefore be the adsorbed lyase that contributes to the high recovery rate and the decrease in column activity.

PURIFICATION OF AF

1. The lyase-Amylopectin/Soluble Starch System

In this system, the reaction system contained AF, limit dextrin, the lyase, and buffer salts at the end of the reaction. AF was separated from the macromolecules (limit dextrin and the lyase) by ethanol (final conc. 50%) precipitation. Unprecipitated low-

molecular-weight amylopectin was separated by ultrafiltration using Amicon YM3 membranes (cut-off 3,000). Ethanol was removed by evaporation at 40°C in a rotary evaporator. Buffer salts were removed from AF by mixed ion exchangers. Purified solid AF was obtained by freeze-drying.

5

2. The Lyase-Pullulanase/Amylopectin/Soluble Starch System.

In this system the final products are AF and glucose. If at least a substantially pure sample of AF is to be prepared, the by-product glucose must be removed. This can
10 be achieved by enzymatic methods. First the glucose is converted into gluconic acid and hydrogen peroxide by glucose oxidase.

Catalase is needed to dispel H_2O_2 formed. H_2O_2 will oxidize AF into two new compounds which are at present of unknown structure. The other impurities in the AF
15 preparation are the oxidation products of AF. It was observed that AF can slowly be oxidized by air-level of oxygen, especially at high temperature, high AF concentration and long time of exposure. Gluconic acid was removed together with the buffer salts by ion exchange chromatography.

20 In this system, the low-molecular-weight amylopectin molecules may alternatively be hydrolysed by amyloglucosidase instead of using ultrafiltration.

3. The purity checking of AF.

25 The purity of the AF preparations were confirmed by TLC, Dionex and NMR.

4. Analysis of the antioxidative activity of anhydro fructose.

Electrochemical oxygen consumption:

30

The activity of AF was investigated in a methyl linoleate emulsion as described by Jorgensen and Skibsted (Z. Lebensm. Unters. Forsch. (1993) 196: 423-429) with

minor modifications: To 5.00 ml of a 1.33 mM methyl linoleate emulsion in 5.0 mM aqueous phosphate buffer with pH = 5.8 and 0.2 w/w % Tween 20 as emulsifier was added AF in the following concentrations: 0, 15, 146 and 680 μ M. The oxidation in the system was initiated by addition of 50 μ l 0.26 M metmyoglobin (MMb) final concentration 0.26 mM. Immediately after initiating the reaction the sample was injected to a thermostated ($25.0 \pm 0.1^\circ\text{C}$) 70 μ l closed cell, effectively excluding diffusion of oxygen into the system. The oxygen consumption was measured by a Clark electrode, which was connected to a PC data collection program. The relative oxygen concentration (%) was registered every 30s.

The results of the experiments produced curves corresponding to oxygen consumption. For samples without addition of AF a relative decrease in oxygen concentration is seen immediately after injection of the sample. For samples containing AF a lag-phase is observed before the curve breaks off and the oxygen concentration is reduced. After the lag-phase only a minor reduction in the oxygen consumption rate is observed compared to samples without AF added. A tendency for samples having the highest amount of AF to have the longest lag-phase is observed. Also, the rate for oxygen consumption is lower for these samples, which was seen by the smaller slope of the curves compared to the slope for the references (0 μ M).

ESR analysis:

Hydroxyl radicals were generated by a Fenton reaction with H_2O_2 (0.17 mM) and FeSO_4 (4.8 μ M). The generated radicals were trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 9.7 mM). AF was added in concentrations of 1.3 mM and 6.3 mM. A water soluble extract of rosemary (*Rosmarinus officinalis* L.) was analyzed in a concentration of 0.25 mg/ml (in grams equivalent to 1.26 mM AF). Measurements were carried out at room temperature ($20 \pm 1^\circ\text{C}$) after 120 s and repeated for the same reaction mixture after 300 s with the following spectrometer settings: Center field 3475.60 G; sweep width 55 G; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.01 G; receiver gain $1.00 \cdot 10^5$; conversion time 81.92 ms time constant 163.84 ms and sweep time 83.89 s.

The results showed that the generated hydroxyl radicals were trapped by DMPO. The spin adduct gives rise to a characteristic 1:2:2:1 ESR spectrum. The peak height of the spectrum is proportional to the quantitative amount of generated spin adduct. Addition of both DMPO and AF will set up a competition between the spin trap and AF. A reduction of peak height will indicate a good scavenging activity of AF.

Table: Peak height of ESR-spectra. $H_2O_2 = 0.17\text{mM}$ and $Fe^{2+} = 4.8 \mu\text{M}$.

Anhydro fructose [mM]	Rosemary extract [mg/ml]	Peak height [120 s]	Peak height [300 s]
0	0	2475	2780
1.3	0	2634	2545
6.3	0	1781	1900

At a concentration of 1.3 mM AF no scavenging activity of hydroxyl radicals is seen, at 6.3 mM Af the peak height is reduced, indicating that a part of the generated hydroxyl radicals is scavenged by AF.

USE OF AF AS AN ANTI-OXIDANT

1. Use of AF as an anti-oxidant in a 50% mayonnaise.

50% mayonnaise is used for salads, open sandwiches, etc. in both the catering and the retail trades. The low oil content of 50% mayonnaise makes it suitable for low-calorie applications.

A typical mayonnaise composition is as follows:

	Soya oil	50.0%
	Tarragon vinegar (10%)	4.0%
5	Egg yolk	3.5%
	Sugar	3.0%
	Salt	1.0%
	Potassium sorbate	0.1%
	Water	35.2%
10	MAYODAN 602	3.0%
	Lemon flavouring 10251	0.2%

MAYODAN 602 ensures a fine, stable oil dispersion and the required viscosity, thereby providing 50% mayonnaise with a long shelf life.

15

Flavouring 10251 is a natural lemon flavouring which provides mayonnaise with the fresh taste of lemon.

Typically the mayonnaise is prepared by the following method:

20

1) Dry mix the MAYODAN 602, sugar and salt. Disperse in oil in a ratio of 1 part powder to 2 parts oil.

25

2) Add flavouring and potassium sorbate to the water and pour into the Koruma mixer. Add 1).

3) Add the egg yolk.

30

4) Add the oil continuously in a vacuum.

5) After 2/3 of the oil has been added (slowly), blend the tarragon vinegar with the remaining 1/3 of the oil, and add.

The following data show that when AF is added to the mayonnaise as an anti-oxidant the results are comparable to the known food anti-oxidants GRINDOX 142 and GRINDOX 1029.

5 GRINDOX 142:

	Ascorbyl palmitate	10%
	Propyl gallate	20%
	Citric acid	10%
	Food grade emulsifier	60%
10	Form at 25°C	paste
	Colour	grey to pale brown
	Density	1.1 g/ml

(All percentages are by weight)

15 GRINDOX 1029:

	Ascorbyl palmitate	20%
	Natural tocopherols	20%
	Food grade emulsifier	60%
	Form at 25°C	paste
20	Colour	light brown
	Density at 25°C	1,0 g/ml

(All percentages are by weight)

25 In the test procedure the anti-oxidants were added to the mayonnaise to provide an anti-oxidant concentration in the order of about 500 ppm. The mayonnaise was then placed in a bomb calorimeter at temperature 80°C containing pure O₂. An induction period to the onset of substantial oxidation of the product is then measured.

30 The results were as follows.

Samples:		IP (hours)
1.	Blank	28,0
2.	+ 500 ppm GRINDOX 142	35,0
3.	+ 500 ppm GRINDOX 1029	33,3
5 4.	+ 550 ppm GRINDOX 1029	34,3
5.	+ 500ppm 1,5 anhydro-D-fructose	32,0

(IP hours = Induction Period)

10 These results show that AF is an excellent food anti-oxidant and is comparable with the known foodstuffs anti-oxidants GRINDOX 142 or GRINDOX 1029.

2. Use of AF as an anti-oxidant in a salad dressing

YOGURT SALAD DRESSING WITH 50% OIL

15

Yogurt salad dressing with 50% oil is used for salads, potatoes, raw vegetable salad, meat, fish and boiled vegetables.

Composition

20	Soya oil	50.0%
	Yogurt (plain)	39.0%
	Vinegar (10%)	3.5%
	Sugar	3.0%
	Egg yolk	2.0%
25	Salt	1.0%
	Potassium sorbate	0.1%
	MAYODAN 525	1.4%
	Acid masking flavouring 2072	0.02%

30 MAYODAN 525 provides unique emulsion stability, prevents syneresis, ensures uniform oil dispersion and viscosity, improves tolerance to production processes and ensures a long shelf life.

40

Flavouring 2072 is a nature-identical, acid masking flavouring reducing the acidulated taste of dressing without affecting its pH value.

Method

- 5 1. Dry mix MAYODAN 525, sugar and salt. Disperse in oil in a ratio of 1 part powder to 2 parts oil.
2. Fill flavouring, potassium sorbate and yogurt into the Koruma mixer. Add 1).
3. Add the egg yolk.
- 10 4. Add the oil continuously in a vacuum.
5. After 2/3 of the oil has been added (slowly), blend the vinegar with the remaining 1/3 of the oil, and add.
6. Add spices if required.

15 Test results:

Sample:	<u>IP hours</u>	<u>PF</u>
1. Blank	37.2	1.00
2. 500 ppm anhydrofructose	39.5	1.06
20 3. 800 ppm GRINDOX 1032	43.3	1.07

(IP – Induction Period); (PF – Protection Period)

Protection Factor (PF):

For each temperature defined as

25 PF = IP of the oil with added antioxidant/IP of the same oil without added antioxidant

Life extension (LE) %:

$$LE = (PF - 1.0) \times 100$$

30

FURTHER STUDIES

1. Effect of alcohols in increasing the activity and stability of the lyase purified from the algae.

5

1-propanol, 2-propanol and 1-butanol were tested at the following concentrations (0%, 1%, 5% and 10%). The optimal concentration of 1-propanol was 5% which increased the AF yield by 34% after 6 days of incubation; the optimal concentration for 2-propanol was 1% which increased the AF yield by 20% after 10 days incubation; the optimal concentration for 1-butanol was 5% which increased the AF yield by 52% after 3-day incubation.

10

Ethanol was tested at the following concentrations (0, 1, 3, 5, 7, 9, 11, 13, 15%). The optimal concentration for 7 days incubation was 5% which increased the AF yield by 12%. For 10 days incubation the optimal concentration was 3% which increased AF yield by 16%.

15

The effect of 1-propanol:

20

1-propanol concentration (v/v)	Reaction time (days)				
	0	1	3	6	10
	AF yield (μ mol)				
0%	0	84	261	451	689
1%	0	80	280	530	803
5%	0	115	367	605	853
10%	0	107	307	456	583

25

30

2. Effect of different reaction media upon the production of AF by the lyase purified from the algae

The results (see table below) indicate that the best reaction medium is 5 mM of HOAc-NaOAc (pH 3.9) (BACE for short) and containing mM concentrations of Na₂-EDTA. The production of AF using either pure water or 0.85% NaCl as reaction medium decreased the yield. Inclusion of 0.85% of NaCl in BACE also decreased the AF yield.

	Reaction Media	Reaction Time (days)			
		0	1	3	8
10		AF yield (μ mol)			
	BACE	0	229	498	575
	Water	0	46	128	217
	NaCl (0.85%)	0	123	239	249
15	BACE+NaCl (0.85%)	0	153	281	303

The following buffers: Mes-NaOH, Mops-NaOH, Hepes-NaOH, and Bicine-NaOH were the optimal reaction media for the lyase.

20

3. The effect of endoamylases and debranching enzymes upon the AF production.

Endoamylase

25 The starch used for AF production may first be liquified either by endoamylases, or by acid hydrolysis.

30 Endoamylase degraded starch is more suitable as substrate for the lyase as compared to native starch. Starch has a limited solubility at the temperature used for the lyase-catalyzed reaction. Treatment of starch with endoamylases led to increased glucose yield. It was found that a reducing matter of around 10-15% (on a dry matter basis) was most suitable as substrate for the lyase with respect to AF yield and further

treatment with the endoamylase to a reducing matter of 19% was no longer suitable for the lyase.

Pullulanase and isoamylase

5

As seen from the results below, both the isoamylase and the pullulanase increased AF yield by up to 50% at pH 4.5 and 5.0. The reaction system consisted of the lyase from the algae with or without the addition of isoamylase or pullulanase (MegaZyme Ltd.). Amylopectin was used as substrate. The AF produced in the presence of only the lyase was expressed as 100%.

10

The pH of the reaction medium

Enzymes added	The pH of the reaction medium		
	3.5	4.5	5.0
Lyase only	100	100	100
Lyase + isoamylase	136	152	150
Lyase + pullulanase	132	158	155

15

20

4. Reversible and Irreversible Inhibitors of the lyase

The reversible inhibitors, Glucose and Maltose.

25

At a substrate concentration of 10mg/ml, the activity for the lyase decreased by 19.3 % in the presence of 0.1 M glucose when amylopectin was used as substrate; the activity was not affected when glycogen was used as substrate. In the presence of 0.1 M of maltose the activity decreased by 48.8 % and 73.4%, respectively for glycogen and amylopectin.

30

44

	Substrates	Inhibitors	
	Concentrations	Glucose	Maltose
5	Amylopectin 1% (2%)	19.3%(7%)	73.4% (67.2%)
	Glycogen 1% (2%)	0.000 (-)	48.8% (49.7%)

10 It seems that the inhibition by 0.1 M glucose is competitive as increasing the substrate from 1% to 2% decreased the inhibition from 19.3 to 7%, whereas the inhibition by 0.1 M maltose is non-competitive as the increase of substrate did not significantly affect the inhibition degree.

	Substrates	Glucose	Maltose
15	Amylopectin (1%)	28%	80%
	Glycogen (1%)	5%	57%

20

The reversible inhibitor deoxyjirimycin

25 At a final substrate concentration of 2%, the activity was decreased to 10.4% for the algal lyase in the presence of 25 μ M of deoxyjirimycin, using amylopectin as substrate. At 100 μ M, the activity of the lyase was completely lost.

Irreversible Inhibitor: PCMB

30 Under the same assay conditions and in the presence of 2 mM PCMB, the activity decreased by 60% for the lyase.

SCALE PRODUCTION OF AF

1. Production of AF using dextrin as substrate

5 The reactor contained 1000 g dextrans (obtained by treatment of starch with Termamyl to a final reducing matter of 10 %) in a final volume of 4.6 liter (HOAC-NaOAC, pH 3.9, containing 5 mM Na₂-EDTA). The reaction was initiated by adding 3 mg purified lyase. The reaction was performed at room temperature. At day 19, another batch of lyase (4 mg) was added.

10

Reaction time (days)

0 1 7 13 19 24 31

15

AF produced (grams)

0 18 116 195 264 500 668

2. Using ¹⁴C-Starch for the production of ¹⁴C-AF

20

The uniformly labelled ¹⁴C-starch (340 μCi obtained from Sigma) was vacuum-dried to remove the ethanol it contained and then dissolved in 2 ml water. The reaction was initiated by adding 20 μl purified lyase and 20 μl pullulanase (MegaZyme Ltd.) The reaction was performed overnight at 30 °C. At the end of the reaction, the reaction mixture was filtered using a filter with a molecular mass cut off of 10,000 to remove the enzymes and unreacted starch molecules.

25

The filtrate was applied on a Ca₂ carbohydrate column (Chrompack) using a Waters HPLC. Water was used as eluent. The flow rate was 0.5 ml/min. AF was efficiently separated from glucose and maltosaccharides. The pooled AF fractions were freeze-dried and totally 140 μCi ¹⁴C-AF was obtained.

30

These findings relate to an even further aspect of the present invention, namely the use of a reagent that can increase the hydrophobicity of the reaction medium (preferably an alcohol) to increase the stability and activity of the lyase according to the present invention. This increased stability leads to a increased AF yield.

5

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

10

15

20

25

30

[GRINDOX as used herein is a trade mark. MAYODAN as used herein is a trade mark.]

SEQUENCE LISTING

(1) GENERAL INFORMATION

NAME OF APPLICANTS: DANISCO A/S
BUSINESS ADDRESS: Langebrogade 1
DK-1001 Copenhagen K
Denmark

TITLE OF INVENTION: ENZYME

SEQ. ID. NO. 1

SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: ALGAL
SEQUENCE LENGTH: 3279 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

	10	20	30	40	50	60
1	ATGTTTCCTA	CCCTGACCTT	CATAGCGCCC	AGCGCGCTGG	CCGCCAGCAC	CTTTGTGGGC
61	GCGGATATCC	GATCGGGCAT	TCGCATTCAA	TCCGCTCTTC	CGGCCGTGCG	CAACGCTGTG
121	CGCAGGAGCA	AACATTACAA	TGTATCCATG	ACCGCATTGT	CTGACAAGCA	AACCGCTATC
181	AGTATTGGCC	CTGACAATCC	GGACGGTATC	AACTACCAAA	ACTACGATTA	CATCCCTGTA
241	GCGGGCTTTA	CGCCCCTCTC	CAACACCAAC	TGGTATGCTG	CCGGCTCTTC	CACTCCGGGC
301	GGCATCACCG	ACTGGACCGC	TACCATGAAT	GTCAAATTCG	ACCGCATTGA	CAATCCGTCG
361	TACTCCAATA	ACCATCCTGT	TCAGATTCAG	GTCACGTCGT	ACAACAACAA	CAGCTTCAGG
421	ATTGCTTCA	ACCCTGATGG	CCCCATTTCG	GACGTCTCTC	GAGGACCTAT	CCTGAAACAG
481	CAACTCACTT	GGATTCGAAA	CCAGGAGCTG	GCGCAGGGAT	GTAATCCGAA	CATGAGCTTC
541	TCTCCTGAAG	GTTTTTTGTC	TTTTGAAACC	AAAGACCTAA	ACGTTATAAT	CTACGGCAAC
601	TGCAAGATGA	GAGTCACGAA	GAAGGATGGC	TACCTCGTCA	TGGAGAATGA	CGAGTGCAAC
661	TCGCAATCAG	ATGGCAATAA	GTGTAGAGGA	TTGATGTACG	TTGACCGGCT	ATACGGTAAT
721	GCTATTGCTT	CCGTACAAAC	GAATTTTCAC	AAAGACACTT	CTCGGAACGA	GAAATTCTAT
781	GGTGCAGGTG	AAGTCAACTG	TCGCTATGAG	GAGCAGGGTA	AGGCGCCGAC	TTATGTTCTA
841	GAACGCTCTG	GACTCGCCAT	GACCAATTAC	AATTACGACA	ACTTGAAC TA	CAACCAACCA
901	GACGTCGTTT	CTCCAGGTTA	TCCCGACCAT	CCCAACTACT	ACATTCCAAT	GTACTACGCA
961	GCACCGTGGT	TGGTCGTTCA	GGGATGCCCG	GGGACATCGA	AGCAATACTC	GTACGGTTGG

1021 TTTATGGACA ATGTCTCTCA GTCGTACATG AACACTGGAG ATACGGCGTG GAACTGCGGA
1081 CAGGAAAACC TGGCATACAT GGGCGCGCAA TACGGGCCAT TTGATCAGCA CTTTGTGTAT
1141 GGTGATGGAG ATGGCCTTGA AGATGTCGTC AAAGCGTTCT CCTTCTTCA AGGAAAGGAG
1201 TTCGAAGACA AAAAATCAA CAAGCGTTCT GTAATGCCTC CGAAGTACGT GTTTGGTTTC
1261 TTCCAGGGTG TTTTCGGTGC ACTTTCCTG TTGAAGCAGA ATCTGCCTGC CGGAGAGAAC
1321 AACATCTCAG TGCAAGAGAT TGTGGAGGGT TACCAGGATA ACGACTACCC CTTTGAAGGG
1381 CTCGCGGTAG ATGTTGATAT GCAAGATGAT CTGCGAGTGT TTACTIONAA ACCAGAATAT
1441 TGGTCGGCAA ACATGGTAGG CGAAGGCGGT GATCCTAATA ACAGATCACT CTTTGAATGG
1501 GCACATGACA GGGGCCTTGT CTGTCAGACG AACGTAACCT GCTTCTTGAG GAACGATAAC
1561 AGTGGGAAAC CATACGAAGT GAATCAGACA TTGAGGGAGA AACAGTTGTA TACGAAGAAT
1621 GATTCCTTGA ACAACACCGA TTTTGAACT ACCTCGGATG GGCCTGGCGA TCGGTACATT
1681 GGACATTTGG ACTATGGTGG TGGAGTGGAG TGTGATGCAA TCTTCCCAGA CTGGGGTCGA
1741 CCAGACGTGG CTCAATGGTG GGGAGAAAAC TACAAGAAGC TGTTCAGCAT TGGTCTCGAT
1801 TTCGTGTGGC AGGATATGAC GGTACCTGCG ATGATGCCGC ACCGACTCGG TGATGCTGTC
1861 AACAAAAATT CCGGTAGTTC GGGCCCGGGC TGGCCGAATG AGAACGATCC ATCCAACGGA
1921 CGATACAACT GGAAATCTTA TCATCCGCAA GTGCTCGTGA CCGACATGCG CTATGGTGCA
1981 GAGTATGGAA GGGAAACGAT GGTGTCTCAA CGCAACATTC ACGCCTACAC TCTTTGTGAA
2041 TCTACCAGAC GGGAGGGAAT TGTGGGAAAC GCAGACAGTT TGACCAAGTT CCGCCGCACT
2101 TACATCATCA GTCGAGGAGG TTACATCGGT AACCAGCATT TCGGAGGGAT GTGGGTTGGG
2161 GACAACAGTG CCACAGAATC CTACCTCAA ATGATGTTGG CGAACATTAT CAACATGAAT
2221 ATGTCGTGCC TCCCGCTAGT TGGCTCTGAT ATTGGCGGGT TCACCCAGTA CAATGATGCG
2281 GGCGACCCAA CCCCCGAGGA TTTGATGGTA AGATTCTGTC AGGCTGGCTG TCTGCTACCG
2341 TGGTTCAGAA ACCACTATGA CAGGTGGATT GAGTCCAAGA AGCACGGGAA GAAATACCAG
2401 GAGTTATACA TGTACCCGGG GCAAAAGGAT ACGTTGAAGA AGTTCGTTGA ATTCCGCTAC
2461 CGCTGGCAGG AGGTTTTGTA CACAGCCATG TACCAAAATG CTACCACTGG AGAGCCGATC
2521 ATCAAGGCGG CGCCCATGTA CAACAACGAC GTCAACGTGT ATAAATCGCA GAATGATCAT
2581 TTCCTTCTCG GTGGACATGA CGGCTATCGT ATTCTCTGCG CACCTGTTGT GCGCGAAAAT
2641 GCGACAAGTC GCGAAGTGTA CCTGCCTGTG TATAGCAAGT GGTCAAATT CGGACCGGAC

2701 TTTGACACTA AGCCCTTGGA AAATGAGATT CAAGGAGGTC AGACGCTTTA TAATTACGCT
2761 GCACCGGTGA ACGATTGCCC GATATTTGTG AGGGAAGGGA CTATTCTTCC GACACGGTAC
2821 ACGCTGGACG GTGTGAACAA ATCTATCAAC ACGTACACAG ACAATGATCC GCTTGTATTT
2881 GAGCTGTTCC CTCTCGAAAA CAACCAGGCG CATGGCTTGT TCTATCATGA TGATGGCGGT
2941 GTCACCACCA ACGCTGAAGA CTTTGCCAAG TATTCTGTGA TCAGTGTGAA GGCCGCGCAG
3001 GAAGGTTCTC AAATGAGTGT CAAGTTTGAC AATGAAGTTT ATGAACACCA ATGGGGAGCA
3061 TCGTTCTATG TTCGTGTTCC TAATATGGGT GCTCCGTCTA ACATCAACGT ATCTTCTCAG
3121 ATTGGTCAAC AGGACATGCA ACAGAGCTCC GTGAGTTCCA GGGCGCAAAT GTTCACTAGT
3181 GCTAACGATG GCGAGTACTG GGTTGACCAG AGCACGAACT CGTTGTGGCT CAAGTTGCCT
3241 GGTGCAGTTA TCCAAGACGC TGCGATCACT GTTCGTTGA

SEQ. ID. NO. 2

SEQUENCE TYPE: NUCLEIC ACID

MOLECULE TYPE: DNA (GENOMIC)

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 1712 BP

STRANDEDNESS: DOUBLE

SEQUENCE:

	10	20	30	40	50	60
1	ATGACAAACT	ATAATTATGA	CAATTTGAAC	TACAATCAAC	CGGACCTCAT	CCCACCTGGC
61	CATGATTCAG	ATCCTGACTA	CTATATTCCG	ATGTACTTTG	CGGCACCATG	GGTGATCGCA
121	CATGGATATC	GTGGCACCAG	CGACCAGTAC	TCTTATGGAT	GGTTTTTGGA	CAATGTATCC
181	CAGTCTTACA	CAAACACTGG	CGATGATGCA	TGGGCTGGTC	AGAAGGATTT	GGCGTACATG
241	GGGGCACAAT	GTGGGCCTTT	CGATCAACAT	TTTGTGTATG	AGGCTGGAGA	TGGACTTGAA
301	GACGTTGTGA	CCGCATTCTC	TTATTTGCAA	GGCAAGGAAT	ATGAGAACCA	GGGACTGAAT
361	ATACGTTCTG	CAATGCCTCC	GAAGTACGTT	TTCGGATTTT	TCCAAGGCGT	ATTCCGAGCC
421	ACATCGCTGC	TAAGGGACAA	CTTACCTGCC	GGCGAGAACA	ACGTCTCTTT	GGAAGAAATT
481	GTTGAAGGAT	ATCAAAATCA	GAACGTGCCA	TTTGAAGGTC	TTGCTGTGGA	TGTTGATATG
541	CAAGATGACT	TGAGAGTGTT	CACTACGAGA	CCAGCGTTTT	GGACGGCAAA	CAAGGTGGGG
601	GAAGGCGGTG	ATCCAAACAA	CAAGTCAGTG	TTTGAGTGGG	CACATGACAG	GGGCCTTGTC
661	TGCCAGACGA	ATGTAACITG	CTTCTTGAAG	AACGAGAAAA	ATCCTTACGA	AGTGAATCAG

50

721 TCATTGAGGG AGAAGCAGTT GTATACGAAG AGTGATTTCCT TGGACAACAT TGATTTTGGG
 781 ACTACTCCAG ATGGGCCTAG CGATGCGTAC ATTGGACACT TAGACTACGG TGGTGGTGTG
 841 GAGTGTGATG CACTATTCCC AGACTGGGGT CGACCAGACG TGGCTCAATG GTGGGGCGAT
 901 AACTACAAGA AACTATTTCAG CATTGGTCTC GATTTCGTCT GGCAAGATAT GACGGTACCT
 961 GCGATGATGC CGCACCGACT CGGTGACCCT GTCGGCACAA ATTCCGGTGA GACGGCGCCG
 1021 GGCTGGCCGA ATGATAAGGA TCCATCCAAC GGACGATACA ATTGGAAGTC TTACCATCCG
 1081 CAAGTGCTCG TGA CTGACAT GAGGTATGAC GATTACGGAA GAGATCCCAT TGTACGCAA
 1141 CGCAATCTCC ATGCCTACAC TCTTTGTGAG TCTACTAGGA GGAAGGCAT TGTGGAAAC
 1201 GCAGATAGTC TGACGAAGTT CCGCCGCAGC TATATTATCA GTCGTGGAGG CTACATCGGT
 1261 AATCAGCACT TTGGTGGGAT GTGGGTAGGA GACAACTCTT CTACGGAAGA CTACCTCGCA
 1321 ATGATGGTTA TCAACGTTAT CAACATGAAC ATGTCCGGTG TCCCGCTCGT TGGTTCCGAT
 1381 ATTGGAGGTT TCACGGAGCA TGACAAGAGA AACCCTTGCA CACCGGACTT GATGATGAGA
 1441 TTTGTGCAGG CTGGATGCTT GCTACCGTGG TTCAGGAACC ACTACGATAG GTGGATCGAG
 1501 AGCAAGAAAC ACGGAAAGAA CTACCAAGAG TTGTACATGT ACCGCGACCA CTTGGACGCC
 1561 TTGAGAAGTT TTGTGGAAC CCGCTATCGC TGGCAGGAAG TGTATACAC AGCCATGTAT
 1621 CAGAATGCTT TGAACGGGAA GCCGATCATC AAAACGGTCT CCATGTACAA CAACGATATG
 1681 AACGTCAAAG ATGCTCAGAA TGACCACTTC CT

SEQ. ID. NO. 3

SEQUENCE TYPE: ENZYME

MOLECULE TYPE: AMINO ACID

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 1092 AMINO ACIDS

SEQUENCE:

				5					10					15
1	Met	Phe	Pro	Thr	Leu	Thr	Phe	Ile	Ala	Pro	Ser	Ala	Leu	Ala
16	Ser	Thr	Phe	Val	Gly	Ala	Asp	Ile	Arg	Ser	Gly	Ile	Arg	Ile
31	Ser	Ala	Leu	Pro	Ala	Val	Arg	Asn	Ala	Val	Arg	Arg	Ser	Lys
46	Tyr	Asn	Val	Ser	Met	Thr	Ala	Leu	Ser	Asp	Lys	Gln	Thr	Ala
61	Ser	Ile	Gly	Pro	Asp	Asn	Pro	Asp	Gly	Ile	Asn	Tyr	Gln	Asn
76	Asp	Tyr	Ile	Pro	Val	Ala	Gly	Phe	Thr	Pro	Leu	Ser	Asn	Thr

91 Trp Tyr Ala Ala Gly Ser Ser Thr Pro Gly Gly Ile Thr Asp Trp
106 Thr Ala Thr Met Asn Val Lys Phe Asp Arg Ile Asp Asn Pro Ser
121 Tyr Ser Asn Asn His Pro Val Gln Ile Gln Val Thr Ser Tyr Asn
136 Asn Asn Ser Phe Arg Ile Arg Phe Asn Pro Asp Gly Pro Ile Arg
151 Asp Val Ser Arg Gly Pro Ile Leu Lys Gln Gln Leu Thr Trp Ile
166 Arg Asn Gln Glu Leu Ala Gln Gly Cys Asn Pro Asn Met Ser Phe
181 Ser Pro Glu Gly Phe Leu Ser Phe Glu Thr Lys Asp Leu Asn Val
196 Ile Ile Tyr Gly Asn Cys Lys Met Arg Val Thr Lys Lys Asp Gly
211 Tyr Leu Val Met Glu Asn Asp Glu Cys Asn Ser Gln Ser Asp Gly
226 Asn Lys Cys Arg Gly Leu Met Tyr Val Asp Arg Leu Tyr Gly Asn
241 Ala Ile Ala Ser Val Gln Thr Asn Phe His Lys Asp Thr Ser Arg
256 Asn Glu Lys Phe Tyr Gly Ala Gly Glu Val Asn Cys Arg Tyr Glu
271 Glu Gln Gly Lys Ala Pro Thr Tyr Val Leu Glu Arg Ser Gly Leu
286 Ala Met Thr Asn Tyr Asn Tyr Asp Asn Leu Asn Tyr Asn Gln Pro
301 Asp Val Val Pro Pro Gly Tyr Pro Asp His Pro Asn Tyr Tyr Ile
316 Pro Met Tyr Tyr Ala Ala Pro Trp Leu Val Val Gln Gly Cys Ala
331 Gly Thr Ser Lys Gln Tyr Ser Tyr Gly Trp Phe Met Asp Asn Val
346 Ser Gln Ser Tyr Met Asn Thr Gly Asp Thr Ala Trp Asn Cys Gly
361 Gln Glu Asn Leu Ala Tyr Met Gly Ala Gln Tyr Gly Pro Phe Asp
376 Gln His Phe Val Tyr Gly Asp Gly Asp Gly Leu Glu Asp Val Val
391 Lys Ala Phe Ser Phe Leu Gln Gly Lys Glu Phe Glu Asp Lys Lys
406 Leu Asn Lys Arg Ser Val Met Pro Pro Lys Tyr Val Phe Gly Phe
421 Phe Gln Gly Val Phe Gly Ala Leu Ser Leu Leu Lys Gln Asn Leu
436 Pro Ala Gly Glu Asn Asn Ile Ser Val Gln Glu Ile Val Glu Gly
451 Tyr Gln Asp Asn Asp Tyr Pro Phe Glu Gly Leu Ala Val Asp Val
466 Asp Met Gln Asp Asp Leu Arg Val Phe Thr Thr Lys Pro Glu Tyr
481 Trp Ser Ala Asn Met Val Gly Glu Gly Gly Asp Pro Asn Asn Arg
496 Ser Val Phe Glu Trp Ala His Asp Arg Gly Leu Val Cys Gln Thr

511 Asn Val Thr Cys Phe Leu Arg Asn Asp Asn Ser Gly Lys Pro Tyr
526 Glu Val Asn Gln Thr Leu Arg Glu Lys Gln Leu Tyr Thr Lys Asn
541 Asp Ser Leu Asn Asn Thr Asp Phe Gly Thr Thr Ser Asp Gly Pro
556 Gly Asp Ala Tyr Ile Gly His Leu Asp Tyr Gly Gly Gly Val Glu
571 Cys Asp Ala Ile Phe Pro Asp Trp Gly Arg Pro Asp Val Ala Gln
586 Trp Trp Gly Glu Asn Tyr Lys Lys Leu Phe Ser Ile Gly Leu Asp
601 Phe Val Trp Gln Asp Met Thr Val Pro Ala Met Met Pro His Arg
616 Leu Gly Asp Ala Val Asn Lys Asn Ser Gly Ser Ser Ala Pro Gly
631 Trp Pro Asn Glu Asn Asp Pro Ser Asn Gly Arg Tyr Asn Trp Lys
646 Ser Tyr His Pro Gln Val Leu Val Thr Asp Met Arg Tyr Gly Ala
661 Glu Tyr Gly Arg Glu Pro Met Val Ser Gln Arg Asn Ile His Ala
676 Tyr Thr Leu Cys Glu Ser Thr Arg Arg Glu Gly Ile Val Gly Asn
691 Ala Asp Ser Leu Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg
706 Gly Gly Tyr Ile Gly Asn Gln His Phe Gly Gly Met Trp Val Gly
721 Asp Asn Ser Ala Thr Glu Ser Tyr Leu Gln Met Met Leu Ala Asn
736 Ile Ile Asn Met Asn Met Ser Cys Leu Pro Leu Val Gly Ser Asp
751 Ile Gly Gly Phe Thr Gln Tyr Asn Asp Ala Gly Asp Pro Thr Pro
766 Glu Asp Leu Met Val Arg Phe Val Gln Ala Gly Cys Leu Leu Pro
781 Trp Phe Arg Asn His Tyr Asp Arg Trp Ile Glu Ser Lys Lys His
796 Gly Lys Lys Tyr Gln Glu Leu Tyr Met Tyr Pro Gly Gln Lys Asp
811 Thr Leu Lys Lys Phe Val Glu Phe Arg Tyr Arg Trp Gln Glu Val
826 Leu Tyr Thr Ala Met Tyr Gln Asn Ala Thr Thr Gly Glu Pro Ile
841 Ile Lys Ala Ala Pro Met Tyr Asn Asn Asp Val Asn Val Tyr Lys
856 Ser Gln Asn Asp His Phe Leu Leu Gly Gly His Asp Gly Tyr Arg
871 Ile Leu Cys Ala Pro Val Val Arg Glu Asn Ala Thr Ser Arg Glu
886 Val Tyr Leu Pro Val Tyr Ser Lys Trp Phe Lys Phe Gly Pro Asp
901 Phe Asp Thr Lys Pro Leu Glu Asn Glu Ile Gln Gly Gly Gln Thr
916 Leu Tyr Asn Tyr Ala Ala Pro Leu Asn Asp Ser Pr Ile Phe Val

931 Arg Glu Gly Thr Ile Leu Pro Thr Arg Tyr Thr Leu Asp Gly Val
 946 Asn Lys Ser Ile Asn Thr Tyr Thr Asp Asn Asp Pro Leu Val Phe
 961 Glu Leu Phe Pro Leu Glu Asn Asn Gln Ala His Gly Leu Phe Tyr
 976 His Asp Asp Gly Gly Val Thr Thr Asn Ala Glu Asp Phe Gly Lys
 991 Tyr Ser Val Ile Ser Val Lys Ala Ala Gln Glu Gly Ser Gln Met
 1006 Ser Val Lys Phe Asp Asn Glu Val Tyr Glu His Gln Trp Gly Ala
 1021 Ser Phe Tyr Val Arg Val Arg Asn Met Gly Ala Pro Ser Asn Ile
 1036 Asn Val Ser Ser Gln Ile Gly Gln Gln Asp Met Gln Gln Ser Ser
 1051 Val Ser Ser Arg Ala Gln Met Phe Thr Ser Ala Asn Asp Gly Glu
 1066 Tyr Trp Val Asp Gln Ser Thr Asn Ser Leu Trp Leu Lys Leu Pro
 1081 Gly Ala Val Ile Gln Asp Ala Ala Ile Thr Val Arg

Number of amino acid residues: 1092

Amino acid composition (including the signal sequence):

64 Ala	14 Cys	18 His	33 Met	56 Thr
48 Arg	55 Gln	45 Ile	49 Phe	22 Trp
89 Asn	49 Glu	65 Leu	59 Pro	67 Tyr
73 Asp	94 Gly	46 Lys	73 Ser	73 Val

SEQ. ID. NO. 4

SEQUENCE TYPE: ENZYME

MOLECULE TYPE: AMINO ACID

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 570 AMINO ACIDS

SEQUENCE:

		5		10		15
1	Met	Thr	Asn	Tyr	Asn	Tyr
			Asp	Asn	Leu	Asn
				Tyr	Asn	Gln
					Pro	Asp
16	Leu	Ile	Pro	Pro	Gly	His
				Asp	Ser	Asp
				Pro	Asp	Tyr
					Tyr	Ile
					Pro	
31	Met	Tyr	Phe	Ala	Ala	Pro
				Trp	Val	Ile
				Ala	His	Gly
					Tyr	Arg
					Gly	
46	Thr	Ser	Asp	Gln	Tyr	Ser
				Tyr	Gly	Trp
					Phe	Leu
					Asp	Asn
					Val	Ser
61	Gln	Ser	Tyr	Thr	Asn	Thr
				Gly	Asp	Asp
				Ala	Trp	Ala
					Gly	Gln
					Lys	
76	Asp	Leu	Ala	Tyr	Met	Gly
				Ala	Gln	Cys
				Gly	Pro	Phe
					Asp	Gln
					His	
91	Phe	Val	Tyr	Glu	Ala	Gly
				Asp	Gly	Leu
				Glu	Asp	Val
					Val	Thr
					Ala	

106 Phe Ser Tyr Leu Gln Gly Lys Glu Tyr Glu Asn Gln Gly Leu Asn
121 Ile Arg Ser Ala Met Pro Pro Lys Tyr Val Phe Gly Phe Phe Gln
136 Gly Val Phe Gly Ala Thr Ser Leu Leu Arg Asp Asn Leu Pro Ala
151 Gly Glu Asn Asn Val Ser Leu Glu Glu Ile Val Glu Gly Tyr Gln
166 Asn Gln Asn Val Pro Phe Glu Gly Leu Ala Val Asp Val Asp Met
181 Gln Asp Asp Leu Arg Val Phe Thr Thr Arg Pro Ala Phe Trp Thr
196 Ala Asn Lys Val Gly Glu Gly Gly Asp Pro Asn Asn Lys Ser Val
211 Phe Glu Trp Ala His Asp Arg Gly Leu Val Cys Gln Thr Asn Val
226 Thr Cys Phe Leu Lys Asn Glu Lys Asn Pro Tyr Glu Val Asn Gln
241 Ser Leu Arg Glu Lys Gln Leu Tyr Thr Lys Ser Asp Ser Leu Asp
256 Asn Ile Asp Phe Gly Thr Thr Pro Asp Gly Pro Ser Asp Ala Tyr
271 Ile Gly His Leu Asp Tyr Gly Gly Gly Val Glu Cys Asp Ala Leu
286 Phe Pro Asp Trp Gly Arg Pro Asp Val Ala Gln Trp Trp Gly Asp
301 Asn Tyr Lys Lys Leu Phe Ser Ile Gly Leu Asp Phe Val Trp Gln
316 Asp Met Thr Val Pro Ala Met Met Pro His Arg Leu Gly Asp Pro
331 Val Gly Thr Asn Ser Gly Glu Thr Ala Pro Gly Trp Pro Asn Asp
346 Lys Asp Pro Ser Asn Gly Arg Tyr Asn Trp Lys Ser Tyr His Pro
361 Gln Val Leu Val Thr Asp Met Arg Tyr Asp Asp Tyr Gly Arg Asp
376 Pro Ile Val Thr Gln Arg Asn Leu His Ala Tyr Thr Leu Cys Glu
391 Ser Thr Arg Arg Glu Gly Ile Val Gly Asn Ala Asp Ser Leu Thr
406 Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg Gly Gly Tyr Ile Gly
421 Asn Gln His Phe Gly Gly Met Trp Val Gly Asp Asn Ser Ser Thr
436 Glu Asp Tyr Leu Ala Met Met Val Ile Asn Val Ile Asn Met Asn
451 Met Ser Gly Val Pro Leu Val Gly Ser Asp Ile Gly Gly Phe Thr
466 Glu His Asp Lys Arg Asn Pro Cys Thr Pro Asp Leu Met Met Arg
481 Phe Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His Tyr
496 Asp Arg Trp Ile Glu Ser Lys Lys His Gly Lys Asn Tyr Gln Glu
511 Leu Tyr Met Tyr Arg Asp His Leu Asp Ala Leu Arg Ser Phe Val

526 Glu Leu Arg Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr

541 Gln Asn Ala Leu Asn Gly Lys Pro Ile Ile Lys Thr Val Ser Met

556 Tyr Asn Asn Asp Met Asn Val Lys Asp Ala Gln Asn Asp His Phe

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>13</u> . line S <u>1-6</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Culture Collection of Algae and Protozoa (CCAP)	
Address of depositary institution (including postal code and country) Dunstaffnage Marine Laboratory PO Box 3 Oban Argyll PA34 4AD Scotland United Kingdom	
Date of deposit 11 October 1994	Accession Number CCAP 1373/2
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<p style="text-align: center;">For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p style="text-align: right; margin-right: 50px;"><i>R.L.R.</i> R.L.R. PETHER</p> <p>Authorized officer</p>	<p style="text-align: center;">For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>
--	--

CLAIMS

1. An enzyme comprising at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.
2. A nucleotide sequence coding for the enzyme of claim 1.
3. A nucleotide sequence according to claim 2 wherein the nucleotide sequence is a DNA sequence.
4. A nucleotide sequence comprising a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.
5. A method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with the enzyme α -1,4-glucan lyase, characterised in that enzyme is used in substantially pure form and wherein the enzyme is isolated from algae alone.
6. A method according to claim 5 wherein the algae is *Gracilariopsis lemaneiformis*.
7. A method according to claim 5 or claim 6 wherein the enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.
8. A method according to any preceding claim wherein the enzyme is obtained from the expression of a nucleotide sequence coding for the enzyme.
9. A method according to claim 8 wherein the nucleotide sequence is a DNA sequence.
10. A method according to claim 9 wherein the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or

contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

5 11. A method according to any one of claims 5 - 10 wherein if the glucan contains links other than and in addition to the α -1,4- links the α -1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.

10 12. A method according to any one of claims 5 - 11 wherein the glucan is starch and preferably a hydrolase, preferably a glucanohydrolase, is used in conjunction with the α -1,4-glucan lyase.

13. The method according to claim 12 wherein the starch is used in high concentration - such as up to about 25% solution.

15 14. A method according to claim 12 or claim 13 wherein the hydrolase is at least one of pullanase or isoamylase.

20 15. A method according to any one of claims 5 to 14 wherein the α -1,4-glucan lyase is bound to a support or, more preferably, is in a dissolved form.

16. A method according to claim 15 wherein the enzyme is isolated and/or further purified from algae alone using a gel that is not degraded by the enzyme.

25 17. A method according to claim 16 wherein the gel is based on dextrin or derivatives thereof, preferably the gel is a cyclodextrin - more preferably β -cyclodextrin.

18. The method according to any one of claims 5 to 17 wherein the substrate is treated with the enzyme in the presence of a buffer.

30 19. The method according to any one of claims 5 to 17 wherein the substrate is treated with the enzyme in the presence of at least substantially pure water.

20. The method according to any one of claims 5 to 19 wherein the substrate is treated with the enzyme in the absence of a co-factor.
21. The method according to any one of claims 5 to 20 wherein the enzyme is used
5 in combination with amylopectin or dextrin.
22. A method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with the enzyme α -1,4-glucan lyase characterised in that enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3-4,
10 or any variant thereof.
23. The sugar 1,5-D-anhydrofructose when prepared by the method of the present invention.
- 15 24. The use of a reagent that can increase the hydrophobicity of the reaction medium to increase the stability and activity of the GL enzyme.
25. Use of AF prepared by the method of the present invention as an anti-oxidant.
- 20 26. Use of AF prepared by the method of the present invention as a sweetener.

1/2

Fig 1.A

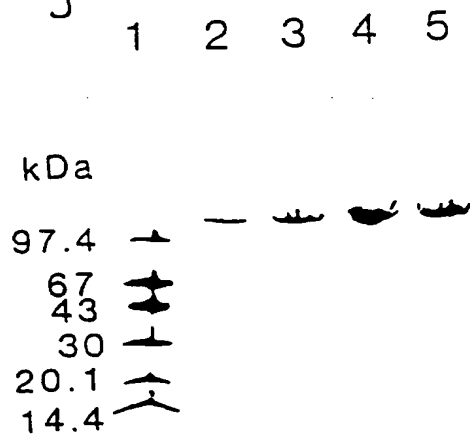
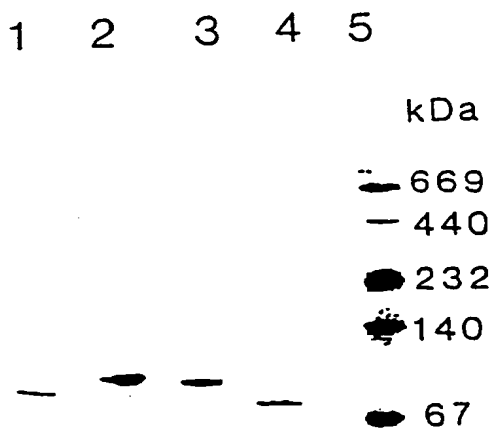
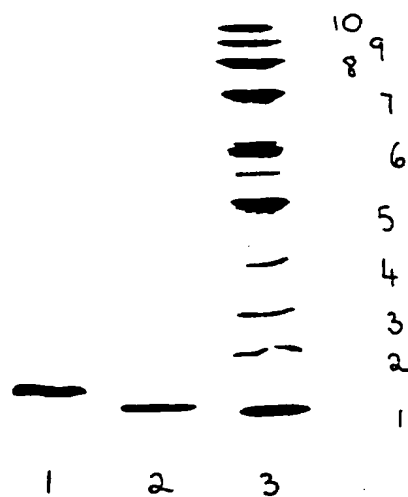


Fig 1.B



2/2

Fig 2



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/02172

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12N9/88 C12P19/02 C07H3/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO,A,94 09122 (ALGATECH AB) 28 April 1994</p> <p>see page 1, line 6 - line 26 see page 2, line 16 - page 3, line 5 see page 3, line 18 - page 5, line 30 see page 8, line 17 - line 25 see page 9, line 1 - line 20 see page 10, line 37 - page 11, line 8 see page 11, line 26 - page 12, line 35 see page 16, line 29 - line 35 see page 28, line 10 - page 30, line 33 --- -/--</p>	<p>5,6, 11-16, 19-21,23</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

13 October 1995

Date of mailing of the international search report

16.01.96

Name and mailing address of the ISA

European Patent Office, P.B. 3818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

MONTERO LOPEZ B.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/02172

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR,A,2 617 502 (SOCIÉTÉ NATIONALE ELF AQUITAINE) 6 January 1989 cited in the application	23
A	see page 2, line 13 - page 3, line 26 see page 4, line 7 - line 35 see page 5, line 16 - page 6, line 26 ---	5,12,15, 19-22
A	BIOCHIMICA ET BIOPHYSICA ACTA, vol.1156, no.3, pages 313 - 320 SHUKUN YU ET AL. 'Alpha-1,4-glucan lyase, a new class of starch/glycogen degrading enzyme. I. Efficient purification and characterization from red seaweeds' cited in the application see abstract see page 314, left column, paragraph 3 - page 315, left column, paragraph 1 see page 314, left column, paragraph 2 - page 315, left column, paragraph 1 see page 315, right column, paragraph 3 - page 316, right column, paragraph 1 ---	1
A	PLANTA, vol.191, no.1, pages 137 - 142 SHUKUN YU ET AL. 'Alpha-1,4-glucan lyase, a new class of starch/glycogen-degrading enzyme' see page 137, left column, paragraph 1 see page 137, right column, paragraph 3; figures 4A,8 ---	1
P,X	WO,A,95 10616 (DANISCO A/S) 20 April 1995 see page 2, line 27 - page 5, line 20 ---	5,6,8,9, 11-21,23
P,X	WO,A,95 10618 (DANISCO A/S) 20 April 1995 see page 1, line 1 - page 4, line 27 -----	5,6,8,9, 15-17

INTERNATIONAL SEARCH REPORT

national application No.

PCT/EP 95/02172

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- 4 subjects - See additional sheet PCT/ISA/210

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-23.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. claims 1-23: 1,5-D-anhydrofructose, method for its preparation,
enzyme therefore and nucleotide sequence encoding it
2. claim 24 : Method to increase stability and activity of Glucan lyase
3. claim 25 : Use of anhydrofructose as anti-oxidant
4. claim 26 : Use of anhydrofructose as sweetener

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 95/02172

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9409122	28-04-94	AU-B- 5347094 EP-A- 0665881 SE-A- 9203084	09-05-94 09-08-95 22-04-94
FR-A-2617502	06-01-89	NONE	
WO-A-9510616	20-04-95	AU-B- 7937994 AU-B- 7856394 WO-A- 9510618 AU-B- 7856294 WO-A- 9510617	04-05-95 04-05-95 20-04-95 04-05-95 20-04-95
WO-A-9510618	20-04-95	AU-B- 7856394 AU-B- 7937994 WO-A- 9510616	04-05-95 04-05-95 20-04-95